Potential Microorganisms for Prevention of Paraffin Precipitation in aHypersaline Oil Reservoir

energy&fuels

Article

pubs.acs.org/EF

Potential Microorganisms for Prevention of Paraffin Precipitation in a Hypersaline Oil Reservoir

Fan Zhang,[†] Yuehui She,*[,‡] Ibrahim M. Banat,[§] Lujun Chai,[†] Shaojing Yi,[‡] Gaoming Yu,[‡] and Dujie Hou*[,†]

[†]The Key Laboratory of Marine Reservoir Evolution and Hydrocarbon Accumulation Mechanism, Ministry of Education, China; School of Energy Resources, China University of Geosciences (Beijing), 29 Xueyuan Road, Haidian, Beijing 100083, China

[‡]College of Chemistry and Environmental Engineering, Yangtze University, Jingzhou, Hubei 434023, China

[§]Faculty of Life and Health Sciences, University of Ulster, Coleraine BT52 1SA, N. Ireland, U.K.

ABSTRACT: Based on 454 pyrosequencing of 16S rRNA gene amplicons, microbial communities in samples collected from seven wells, six of which had positive paraffin deposition reduction and one with negative paraffin deposition reduction upon a microbial treatment designed for the prevention of paraffin precipitation, were analyzed. Microbial communities' structures were significantly different for the samples from the negative well and the positive wells. Microbes affiliated with *Diaphorobacter* belonging to β-Proteobacteria were predominant in the negative well, While γ-Proteobacteria-affiliated microbes of *Pseudomonas* and *Enterobacter* and Firmicute-affiliated *Bacillus* were shared and dominant in the positive wells. Microbes shared in the positive wells could be considered as potential candidates for investigations into microbial paraffin control. In addition, microbial activity of hydrocarbon-degradation and microbial products such as biosurfactants were proposed to be the main potential mechanisms for the microbial treatment for the prevention of paraffin precipitation.

INTRODUCTION

Paraffin is a complex mixture of hydrocarbons containing n-alkane, iso-alkane, and cycloalkane of carbon chain length C16 to C18 and above.[1] Generally, under high temperature–pressure conditions in oil reservoirs, paraffin remains in equilibrium in crude oil. However, when the crude oil is lifted to wellholes or surface, paraffin begins to precipitate from the crude oil forming particles that adheres to the surface of the pipelines due to the reduction of temperature below the wax appearance temperature (WAT) or Cloud Point.[1,2] This paraffin deposition results in blockages, which ultimately causes the increase of exploitation cost for the petroleum industry.

Paraffin problems are generally mitigated through chemical and physical treatments. Chemical methods are to use chemicals and solvents that inhibit the deposition processes by lowering WATs or cloud points or by dissolving and dispersing deposited paraffin.[1−3] Physical methods include thermal fluid washing and pigging. While most of these processes are in use, they all have some disadvantages. Chemical and solvents use is hazardous as well as costly.[1] The use of mechanical scraping processes often entails losses in oil production due to downtime,[2] while heat washing does not work well when the fluid cools down.[4]

Compared with chemical and physical treatments, microbial treatments for the prevention of paraffin deposition may offer a nonhazardous and economically viable strategy. Two main mechanisms are proposed for such a microbial treatment. The first involves the stimulation of in situ hydrocarbon metabolism. On one hand, hydrocarbon-degraders are stimulated to crack long-chain hydrocarbons to short-chain hydrocarbons, resulting in oil with lower viscosities and improved motilities.[5,6] On the other hand, microbial byproducts such as emulsifiers and biosurfactants coupled with hydrocarbon metabolism can alter the physical properties of crude oil such as viscosity, pour point, and cloud point to prevent paraffin from precipitating.[7,8] The second, a relatively new mechanism, involves the formation of bacteria-carrying film. Microbes growing on the wall of pipes can alter the wettability of metal surface and thus eliminate paraffin deposition.[3,9]

The statistic of numerous published field trials supported the effectiveness of independently derived hydrocarbon-degrading inocula and independently tested commercial formulations in lessening of production problems, reduction of operating costs, and increase of oil production.[6] However, none of the studies mentioned so far address the issue of whether an inoculum is required and is active after injected into wellholes or if indigenous microorganisms caused the changes observed in the fields.[6] Therefore, studies carried out to monitor the response of microbes in situ during microbial treatments are quite sought after. Unlike culture-dependent experiments carried out in laboratories, field trials are carried out in the natural underground environments of oil reservoirs in which the conditions for optimal performance is difficult to control. In cases where microorganisms, nutrients, or both were injected, microbes active during field trials were different from those microbes activated in laboratories.[10,11] Practicably, comparing microbial communities in positive wells where microbial treatments work with those in negative wells where microbial treatments do not work during microbial enhanced oil recovery (MEOR) based on culture-independent methods could be an effective strategy to reveal potential and active microbes for microbial paraffin control.

Received: November 27, 2013
Revised: January 28, 2014
Published: January 30, 2014

dx.doi.org/10.1021/ef402329u | *Energy Fuels* 2014, 28, 1191−1197

Table 1. Information on Characteristics of Oil Production, Gross Composition of Crude Oil. and Microbial Treatment of the Seven Treated Oil Wells in the Wangchang Oil Field

characteristics	WY3X7_5	WX11_6	W4X7_2B	WY3X15_2	W4X14_3C	WX9_7	W4X14_6
Produced fluid							
production horizons	Eq3	Eq4	Eq4	Eq3	Eq3	Eq3	Eq4
production depth (m)	1574.0– 1581.0	1555.6– 1567.0	1906.0– 2499.0	1259.6– 1337.0	1399.4– 1406.4	1373.4– 1385.6	1930.2– 1937.0
produced fluid (m^3)	3.7	14	25.9	29.3	33	17.5	28
average watercut (%)	29.2	78.1	88.7	97.7	94.8	70.3	88.2
added fresh water (m^3)	3	4	0	6	2	1	6
salinity (mg/L)	14371	15092	14325	13323	15874	12547	14652
nitrate (mg/L)	159.4	68.2	47.4	34.7	35.1	66.3	36.1
Produced oil							
wax appearance temperature (°C)	47.1	46.9	46.3	47.5	50.2	46.7	49.3
wax content (%)	28.5	23.1	25.1	30.5	22.7	29.2	23.3
saturated hydrocarbon (%)	45.3	59.5	61.3	53.2	49.6	50.3	51.3
aromatic hydrocarbon (%)	26.5	13.4	16.2	20.3	19.4	22.4	20.1
resins (%)	19.4	10.1	15.3	16.2	12.3	20.8	18.9
asphaletene (%)	0.4	0.3	0.3	0.4	0.3	0.4	0.5
Microbial treatment time							
first injection	3/2/2011	13/5/2010	18/5/2010	5/6/2010	11/10/2011	5/9/2009	18/8/2010
last injection	9/1/2012	7/7/2012	15/10/2011	20/9/2011	9/6/2012	26/3/2012	8/4/2011

With the development of molecular biological techniques and detailed high throughput investigations in the dynamics of microbial groups, a clearer picture of resident and potential microbes in oil reservoirs can emerge. In the present study a field trial of microbial treatment of paraffin deposition was carried in the Wangchang oil field (Jianghan, Hubei, China). Using 454 pyrosequencing of 16S rRNA gene amplicons, we analyzed microbial communities in the samples collected from seven treated oil wells (six with a positive response to microbial treatment and one with a negative response to such treatment) to reveal the activity of injected microbes and actually potential microbes during the field trial.

EXPERIMENTAL SECTION

Characteristics of Study Area and Samples. The Wangchang oil field is located in the Jianghan Plain within central China. The oil field produces oil from two major production horizons, Eq4 and Eq3, with a range of production depths between 1200 and 3045 m, and an average in situ temperature of approximately 90 °C. Even in wellholes the temperature of produced liquid is approximately 48 °C. Oil formations are subjected to water-flooding with an average water-cut of 86%. The formation water has high salinity of approximately 30000 mg/L. In order to prevent salt coagulation in wellholes, fresh water is injected into the wellholes of production wells through oil casing annulus to reduce the salinity of the produced fluid below 15000 mg/L. The crude oil in this site is identified as high-wax crude oil with wax content of approximately 25% and an average WAT of approximately 46 °C, which results in blockages in about 80% of total producing wells. In this study, oil/water mixed samples were collected from seven treated wells including six positive wells (WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6) and one negative well (WY3X7_5) in September 6, 2012. The detail information on characteristics of oil production, gross composition of crude oil, and microbial treatment of the seven treated oil wells are shown in Table 1.

Field Trial. Microbial treatments were introduced into the Wangchang oil field for prevention of paraffin precipitation at the end of the year of 2008 and were popularized in March, 2010. The procedure of a general microbial treatment in producing wells was as follows: after pumps were inspected or wellholes were subjected to thermal fluid washing, 200 kg microbial inocula reaching the late exponential growth phase (approximately 6×10^7 cells/mL) was injected into oil casing annulus. This was followed by a daily injection of a 2 kg mixture of KNO_3 and Na_3PO_4 (1:1, W/W) through the oil casing annulus (Figure 1). The microbial inocula consisted of *Bacillus licheniformis* (KF716142) and *Bacillus thermoamylovorans* (KF716140) which were enriched as wax-degrading microorganisms from the water sample collected from the inlet of the three phrase separator in the Wangchang oil field.[12] Normally, the microbial inocula were injected every 6 months.

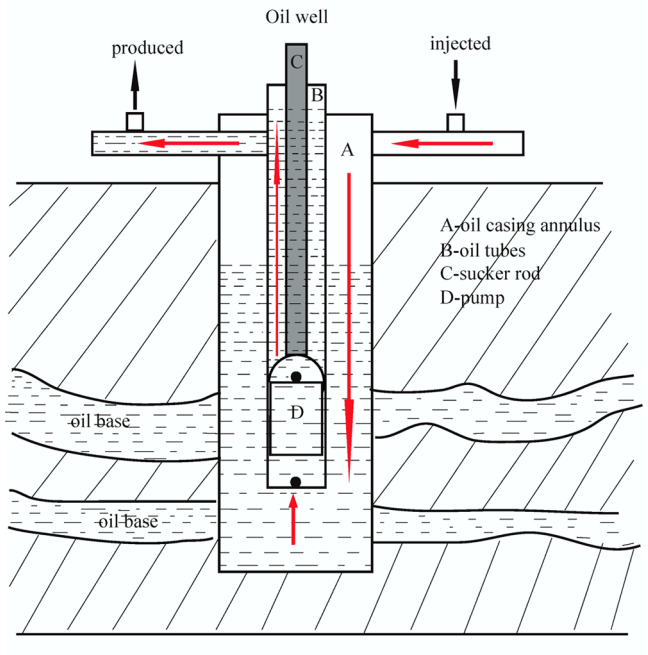

Figure 1. A schematic overview of the microbial treatment for the prevention of paraffin precipitation in a wellhole of a production oil well. Microbial cultures and nutrients were injected directed into oil casing annuluns (A), moved to the bottom of the well and extracted to oil tubes (B) by a pump (D), and then produced to the surface in oil/water mixed liquid.

DNA Extraction. In order to collect cells entrapped in oil, the mixed oil/water samples were warmed at 45–50 °C for 30 min in a

dx.doi.org/10.1021/ef402329u | Energy Fuels 2014, 28, 1191–1197

water bath and then vortex for 10 min. Approximately 150−200 mL of the treated mixed oil/water liquid was centrifuged at 10,000 × g for 10 min to pellet cells. Genomic DNA was extracted from the collected cells following the manufacturer's protocol for the manufacturer's instructions for TIANamp Micro DNA Kit (DP316) (Tian Gen Biotech (Beijing) Co., Ltd., China), and genomic DNA was extracted in triplicate to avoid bias and pooled together for the following analysis. Obtained DNA were purified with an Agarose Gel DNA Purification Kit (TianGen Biotech, Beijing, China).

PCR Amplification, Amplicon Quantitation, Pooling, and Pyrosequencing. 454 pyrosequencing of 16S rRNA gene was performed to identify organisms present in each sample. An approximate 526 bp region covering the V1−V3 region of the 16S rRNA gene of bacteria was amplified with the primers 27F and 533R containing the A and B adaptors (454 Life Science). The forward primer (B-27F) was 5′-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*AGAGTTTGATCCTGGCTCAG-3′, and the reverse primer (A-533R) was 5′-*CCATCTCATCCCTGCGTGTCTCCGACTCAGNNN*NNNNNNNNNNTTACCGCGGCTGCTGGCAC-3′, where the B and A adaptors are the sequences in italics and underlined, and the Ns represent a ten-base sample unique barcode sequence. A 50 μL PCR reacting system performed in triplicate contained 0.6 μM each of the primer, approximately 5 ng of template NDA, 1 × PCR buffer, and 2.5 U of DNA Polymerase (MBI. Fermentas, USA). Negative controls were needed. The amplification program was as follows: an initial denaturation at 94 °C for 4 min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final step of extension of 72 °C for 10 min. After amplification, replicate PCR products of the same sample were pooled and purified using DNA gel extraction kit (Axygen, China).

Before sequencing, the concentration of each PCR product was measured using a Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Germany), and the quality of each PCR product was controlled on an Algilent 2100 bioanalyzer (Agilent, USA). As recommended by 454 Life Science, equimolar rations of each amplicons were mixed in a working pool that was subjected to emulsion PCR to generate amplicon libraries. Amplicon pyrosequencing was performed from the A-end using a 454/Roche A sequencing primer kit on a Roche Genome Sequencer GS FLX Titanium platform (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

Statistical and Bioinformatics Analysis. The raw multiplexed sequence reads were processed according to QIIME-1.6.0 Pipeline (www.qiime.org). Notably, in this step, the data related with OTUs with sequences failed in alignment and unclassified to be bacteria were removed. Hierarchical analysis of columns in the heat map was based on Bray−Curtis similarity and used complete linkage in the R package gplots. In this study, valid representative OTUs were too many to construct a normal phylogenetic tree; we determined technical reproducibility thresholds to conclude that OTUs defined by ≥10 reads in any sample are 'dominant OTUs'. Sequences of dominant OTUs were used to construct a phylogenetic tree using the neighbor-joining method.

RESULTS AND DISCUSSION

Characteristics of the Field Trial. The microbial treatment of prevention paraffin from precipitation in this study was performed in production wells. Microbes and nutrients were injected from surface to oil casing annulus and extracted from oil pipes to surface (Figure 1). Particularly, during the field trial the nutrients were added into the wellholes every day to sustain the growth of potential microbes without stopping the production of oil. Traditionally, oil production during a field trial of paraffin control was shut in for several days to several weeks due to one-off nutrients injection.[6] The apparatus designed for fresh water injection facilitated the injection of nutrients without needing an additional pressurized apparatus during the field trial, which made it convenient to inject nutrients every day. Effectively, approximately 90% of the treated wells showed positive response with an extension of interval time needed for thermal or oil washing by 5 to 22 months. In addition, Figure 2 shows visual comparing pictures

Figure 2. Pictures of sucker rods without and with paraffin deposition from positive (left) and negative (right) wells, respectively. Obviously the effectiveness of a microbial treatment of prevention wax from precipitation is easily measured by detecting paraffin in the surface of sucker-rods.

of sucker rods from positive and negative wells, respectively (the pictures was provided by the Jianghan Oil Field Co., Ltd.), which indicated the effectiveness of the formula of the field trial and implied that stimulated microbes shared and dominated in the wells reacting positively and may therefore be potentially effective in the prevention paraffin from precipitation.

Sequencing Statistics and Diversity Estimates. A total of 46,829 valid reads were obtained and were clustered into 1461 OTUs that were aligned to be bacteria from the seven bacterial communities (WY3X7_5, WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6) through 454 pyrosequencing and bioinformatic analysis. The entire set of the raw reads is available at ENA's Sequence Read Archive (SRA) under accession number of PRJEB4774. The numbers of reads in samples were between 5571 and 7099, with OTUs ranging from 124 to 403. Details on the number of sequences and OTUs from each sample as well as estimates of richness and total diversity calculated at an evolutionary distance of 0.03 are shown in Table 2. The detected high diversity of microbial community indicated that next generation sequencing method of 454 pyrosequencing was more effective in detecting members completely in samples from oil reservoirs than traditional clone library method.[10,11] According to the reproducibility thresholds determined, only 134 OTUs were grouped as dominant OTUs. Notably, although the number of dominant OTUs was only 9.17% (134/1461) of total detected OTU, the abundance of reads clustered into these dominant OTUs in each sample covered a high percentage between 90.9%−97.1% (Table 2), which indicated quantitatively that dominant microbes detected in the samples of produced water appeared with less diversity.

Differences in Predominant Microorganisms Different in the Negatively Responding Well and the Positively Responding Wells. The hierarchical cluster analysis using the R package of gplots provided a preliminary insight into the differences of microbial communities among the samples from the negative well and the positive wells. It was apparent that the microbial community of the negative well (WY3X7_5) presented a long distance from those detected in the positive wells. The plot also showed that the three positive wells samples of WX9_7, W4X14_6, and W4X14_3C were clustered together and then grouped with the other three treated wells samples of WY3X15_2, WX11_6, and W4X7_2B (Figure 3A).

dx.doi.org/10.1021/ef402329u | *Energy Fuels* 2014, 28, 1191−1197

Table 2. Diversity and Richness Indices of Bacteria and Percentage of Dominant OTUs in the Samples from the Seven Treated Oil Wells in the Wangchang Oil Field

	number of reads		$OTU_{0.03}$						
samples	original	dominant[a]	original	dominant[a]	percentage[b] (%)	ACE	Chao	Shannon	coverage (%)
WY3X7_5	7099	6893	124	24	97.1	175	177	0.6	0.99
WX11_6	6777	6282	345	61	92.7	1427	959	3.37	0.95
W4X7_2B	6519	5927	389	73	90.9	1449	886	3.65	0.95
WY3X15_2	6126	5599	402	82	91.4	1526	944	4.17	0.95
W4X14_3C	7364	6701	403	62	91.0	1385	892	3.61	0.95
WX9_7	5571	5202	235	42	93.4	770	493	3.11	0.96
W4X14_6	7373	6938	281	57	94.1	867	6701	3.45	0.96

[a]Dominant represents OTUs defined by ≥10 reads in any sample. [b]Percentage showing the percentage of the reads of dominant OTUs to total number of valid reads detected.

Based on the data of alignment, the microbial communities within the seven samples were dominated by microbes clustered into Proteobacteria and Firmicutes. It was also noted that β-Proteobacteria-affiliated microbes represented an overwhelming percentage of 97.9% in the sample from the negative well, while γ-Proteobacteria-affiliated microbes were dominant in samples collected from the positive wells (88.7%, 14.4%, 53.6%, 81.5%, 91.0%, and 79.9% in WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6, respectively) (Table 3). In addition, Firmicutes-affiliated microbes were present significantly in all the positive wells at 74.2% in the sample of W4X7_2B; this was followed by those in the sample of WY3X15_2 (36.6%), W4X14_6 (17.2%), W4X14_3C (11.9%), W4X11_6 (5.6%), and WX9_7 (5.4%), while only 0.1% Firmicutes-affiliated microbes were detected in the sample from the negative well of WY3X7_5 (Table 3).

To further understand the differences in microbial communities inhabiting the positive wells and the negative well and reveal the potential microbial groups associated with the prevention of paraffin precipitation, we carried out an in-depth analysis of the dominant OTUs in the level of genus in-depth. Figure 3B and 3C show the relationships of dominant microbes in different samples visually. In the sample from the negative well of WX7_5 a total of 6764 reads (95.3%) clustered into 11 dominant OTUs that were all aligned to the genus of *Diaphorobacter* were detected, while *Diaphorobacter*-affiliated microbes covered only a small number of reads of 14, 40, 33, 16, 53, and 34 in the samples from the positive wells of WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6, respectively. To our knowledge *Diaphorobacter* was never detected or isolated in the samples from the environment of oil reservoirs. Strains of *Diaphorobacter* sp. were detected and isolated prevailingly from activated sludge,[13,14] activated biomass,[15] and biofilm[16] as denitrifying microbes for nitrate reduction. As there was no global reported presence of *Diaphorobacter* lineages in oil reservoirs, the possibility of the indigenous nature of *Diaphorobacter* to oil reservoirs was small according to the indication suggested by Youssef et al.[6] Of special focus was, therefore, the origin of *Diaphorobacter* in the samples under study. Besides containments from water-flooding, drilling, and sampling,[6] a possible containment for the introduction of *Diaphorobacter* to the wellholes was the injected nutrients. Unlike microbial lab-grown media, solutions of nutrients during the field trail were injected into wellholes everyday without sterilization, which inevitably introduced microbes habiting in chemicals and water into wellholes. Notably, the concentration of nitrate of the produced fluid from the negative well was much higher than those from the positive wells (Table 1), which seemed to be the key factor resulting in the difference in abundance of *Diaphorobacter* between the negative well and the positive wells based on the functional property of denitrification for strains of *Diaphorobacter*.[13−16]

Microbes belonging to the genus of *Pseudomonas* were dominant and shared in all the samples from the positive wells; the abundance of the genera of *Pseudomonas* in each sample was listed in Figure 3C. Predominantly, the *Pseudomonas*-affiliated OUT 352 covered a number of reads of 1433 (21.4%), 426 (6.5%), 1767 (28.8%), 4023 (54.6%), 3134 (56.4%), and 3639 (49.4%) in the samples from the positive wells of WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6, respectively, while this OTU only occupied a percentage of 0.74% (53 reads) in the sample of the negative well (Figure 3C). The next shared dominant microbial group in the positive wells was microbes affiliated with the genus of *Enterobacter*. Among these *Enterobacter*-affiliated OUTs, only one OTU (OTU 1203) was detected in the sample from the negative well and only occupied 0.2% (14 reads), while *Enterobacter* sp. of this OTU was predominant with a number of reads of 659 (9.7%), 79 (1.2%), 572 (9.3%), 1209 (16.4%), 1279 (23%), and 1360 (18.5%) in the sample from the positive well of WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6, respectively. In fact, members of *Pseudomonas* sp. and *Enterobacter* sp. were not considered as indigenous to oil reservoirs,[6,17] yet numerous studies of microbial community in samples from oil reservoirs reported the presence of *Pseudomonas*.[6,10,11] Members of the *Enterobacter* sp. were frequently detected and isolated from environments associated with oil, such as petroleum waste sludge,[18] solid waste oil samples,[19] oil pipelines,[20] and oil fields,[21] which indicated that the genera of *Pseudomonas* and *Enterobacter* possessed exceptional survival abilities in the environment of oil reservoirs after introduction into wellholes.

The final shared group of microbes in the positive wells was microbes affiliated dominantly with the genus of *Bacillus* and *Geobacillus* clustered into the family of Bacillaceae. This group of microbes occupied 20.6% in the sample of W4X14_6; this is followed by 8.9%, 5.4%, 3.6%, 3.2%, and 1.9% in the samples of W4X14_6, 4X14_3C, W4X7_2B, WY3X15_2, WX9_7, and WX11_6, respectively. In comparison, not a single read affiliated with *Bacillus* was detected in the sample of the negative well. Similarity, it was difficult to determine the indigenous nature of the detected *Bacillus* as there were many factors that could hinder us to make a definite decision.[6] However, strains of *Bacillus* sp. were often used as potential microbes for MEOR.[6]

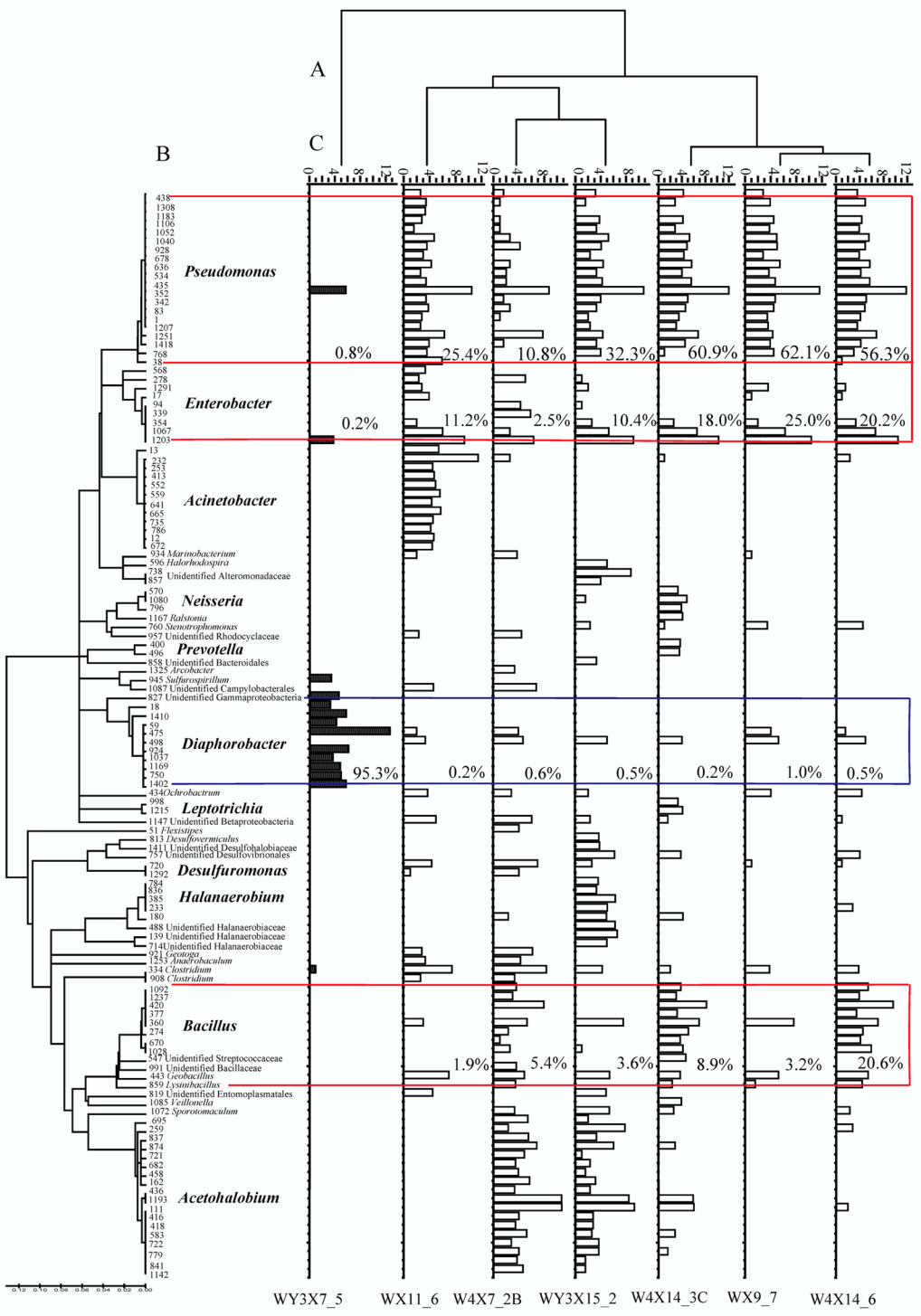

Figure 3. Dominant OTUs alignment analysis of the samples from the negative well of WY3X7_5 and positive wells of WX11_6, W4X7_2B, WY3X15_2, W4X14_3C, WX9_7, and W4X14_6. **A**, Samples and OTUs are clustered on their Bray− Curtis similarities (group-average linkage). **B**, The bacterial phylogenetic tree of the dominant OTUs was constructed using the neighbor-joining method. **C**, Histograms showing the differences in numbers of reads of each dominant OTU detected in each sample. The percentages below the histograms represent the relative abundance of the four dominant groups in each sample. In order to lessen the distance of numbers of maximum and minimum reads, all the numbers of reads were log2 converted.

Microbial communities inhabiting the samples from the negative well and the positive wells were obviously different. Undeniably, heterogeneity of an oil reservoir, connectivity between injection wells with production wells, and different production horizons could result in different microbial structure and physicochemical properties of produced fluids from different oil wells in the same oil reservoir.[10,11,22,23] Therefore, it was possible that original microbes inhabiting the negative well and the positive wells were different before the nutrients were injected. Additionally, active microbes in different

dx.doi.org/10.1021/ef402329u | *Energy Fuels* 2014, 28, 1191−1197

Table 3. Relative Abundance of Dominant Microbes at the Level of Phylum in the Seven Treated Oil Wells in the Wangchang Oil Field

	Proteobacteria (%)									
samples	α	β	γ	δ	ε	Actinobacteria (%)	Bacteroidetes (%)	Deferribacteres (%)	Firmicutes (%)	others (%)
WY3X7_5	0	98.0	1.6	0	0.2	0	0.01	0	0.1	0.1
WX11_6	0.3	1.1	88.7	0.5	0.4	0.2	0.1	0.2	5.6	2.9
W4X7_2B	0.3	2.4	14.4	2.7	2.0	0.3	0.2	0.4	74.2	3.1
WY3X15_2	0.3	1.1	53.6	2.0	0.05	0.2	0.4	1.7	36.6	4.0
W4X14_3C	0.2	1.9	81.5	0.2	0.04	0.7	2.0	0.1	11.9	1.1
WX9_7	0.7	1.3	91.0	0.1	0.02	0.3	0.4	0.13	5.4	0.6
W4X14_6	0.5	1.0	79.9	0.3	0.04	0.3	0.3	0.03	17.2	0.5

wellholes may have varied after nutrients were injected possibly because of the difference in characteristics of produced fluids in different oil wells.[10,11] Nevertheless, high similarity of microbial communities in the samples from the positive wells implied that the shared dominant microbes would possess the same potential ability for microbial paraffin control during the microbial treatment.

In addition, the special lineages with the bacteria community in environments could be best understood by correlating in putative roles of these lineages to the observed geochemical conditions in a location.[24] In this study, the relatively high salinity with all samples (approximately 15000 mg/L) could imply the abundance of lineages that were exclusively halophilic in the bacterial communities within all the samples. However, *Acetohalobium*-affiliated microbes distributed into 18 dominant OTUs were only dominant in two positive wells of W4X7_2B and WY3X15_2 with total reads of 3799 (58.3%) and 1369 (22.4%), respectively. In addition, *Halanaerobium*-affiliated microbes were only dominant in the sample of the negative well of WY3X15_2 (372 reads, 6.1%). Regardless of the influence from the injected nutrients, a significant factor resulting in the decay of halophilic microbes in most of the oil wells under study was the injection of fresh water in the produced wells during the field trial.

Survival of Injected Inocula. Microbes with potential metabolic capabilities isolated in laboratories often were used as inocula in field trials of microbial paraffin control.[6] However, none of studies monitored survival or vigorousness of the injected inocula during field trials. In this study the wax-degrading strains of *Bacillus licheniformis* (KF716142) and *Bacillus thermoamylovorans* (KF716140) were injected to the wells. According to the analysis of microbial communities in the treated wells, few sequences affiliated with *Bacillus licheniformis* and *Bacillus thermoamylovorans* were detected (data set in ENA). Generally, discrepancy in environmental factors for microbial growth between laboratories and natural environments of oil reservoirs was considered as the most important reason for the occurrence of unexpected results during field trials.[10,11] In the present study, one additional reason that must be considered was that fluid production from the production wells was not shut in when the microbial inocula were injected; therefore, microbial inocula possibly were pumped out of the wellholes without abundant proliferation. Therefore, it was suggested that the procedure of inocula injection should be disregarded in the next field trial in the Wangchang oil field.

Potential Microbes and Mechanisms of the Microbial Treatment of Prevention Paraffin from Precipitation. In the present study, *Pseudomonas* sp., *Enterobacter* sp., and *Bacillus* sp. were the three dominant microbial groups appearing in all the samples from the positive wells. *Pseudomonas* sp. and *Bacillus* sp. were reported frequently to be potential microbes for MEOR.[6,11,25,26] It is well-known that strains of *Pseudomonas* and *Bacillus* can produce biosurfactants of lipopetides or rhamnolipids that lower interfacial tension between hydrocarbon and aqueous phases that results in improving mobility of crude oil.[6,27−31] In this study, biosurfactantants produced also could play an important role in altering the wettability of stainless steel and thus eliminate paraffin deposition.[3,9] It is also interesting to note that strains of *Pseudomonas* and *Bacillus* were often reported to degrade alkanes, aliphatic, and aromatic fractions resulting in a decrease of cloud point temperature of crude oil,[32−34] which acts as a significant mechanism of microbial prevention paraffin from precipitation. In this microbial treatment, crude oil was the main carbon source for the growth of microbes to produce biosurfactants in wellholes. Moreover, researchers indicated that biosurfactants produced were capable of promoting biodegradation of crude oil by bacteria in aquatic environments.[35]

Strains of *Enterobacter* have also been implicated in MEOR applications following the mechanism of gas and polymer production,[36,37] which is useful for the MEOR strategy of microbial plugging to improve sweep efficiency. No single strain of *Enterobacter* was reported to be used in microbial prevention paraffin from precipitation. Recently, researchers successively detected and isolated strains of *Enterobacter* from environments associated with crude oil and revealed that most of these strains were biosurfactant-producers that could be used to reduce interfacial tension and alter wettability during MEOR,[18−20,37−42] which was consistent with characteristics reported for strains of *Pseudomonas* and *Bacillus*.

We could not definitely determine what microbial activities and products took place during microbial treatment for the prevention paraffin precipitation. Further isolation and metabolic analysis investigations are therefore required. Microbial community analysis in the negative well and the positive wells could provide an indication of the potential microbes which may be of great importance in guiding further studies concerning the potential application and mechanisms of microbial uses in oil fields.

CONCLUSION

In summary, the microbial communities in the negative well and the positive wells were monitored during the field trial for the prevention paraffin precipitation. Three groups of microbes affiliated to the genera *Pseudomonas*, *Enterobacter*, and *Bacillus* were shared and dominant in all the positive wells and few in the negative well. Microbial activities of hydrocarbon-degraders and microbial products such as biosurfactants were proposed to be the main mechanisms for the microbial treatment for the

prevention paraffin precipitation. The potential microbes detected could serve as reference and base for the further study in microbial prevention paraffin from precipitation.

AUTHOR INFORMATION

Corresponding Authors

*Phone: +86-10-62552493. Fax: +86-10-62552493. E-mail: sheyuehui@263.net (Y.S.).

*E-mail: hdj@cugb.edu.cn (D.H.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was sponsored by the 863 Program (No. 2008AA06Z204) of the Ministry of Science and Technology and the National Natural Science Foundation (No. 50974022 and No. 51074029) in P. R. China. The authors wish to thank the technical personnel in the oil field under study for kindly collecting samples and providing geological information on the oil reservoirs.

REFERENCES

(1) Sood, N.; Lal, B. *Chemosphere* **2008**, *70*, 1445−1451.

(2) Aiyejina, A.; Chakrabarti, D. P.; Pilgrim, A.; Sastry, M. K. S. *Int. J. Multiphase Flow* **2011**, *37*, 671−694.

(3) Xiao, M.; Li, W. H.; Lu, M.; Zhang, Z. Z.; Luo, Y. J.; Qiao, W.; Sun, S. S. *Bioresour. Technol.* **2012**, *124*, 227−232.

(4) Rana, D.; Bateja, S.; Biswas, S.; Kumar, A.; Misra, T.; Lal, B. *SPE* **2010**, 129002.

(5) Brown, F.; Maure, A.; Warren, A. *U.S. Patent Office*. 2005. Patent No. 6905870.

(6) Youssef, N.; Elshahed, M. S.; McInerney, M. J. *Adv. Appl. Microbiol.* **2009**, *66*, 141−251.

(7) Barkay, T.; Navon-Venezia, S.; Ron, E. Z.; Rosenberg, E. *Appl. Environ. Microbiol.* **1999**, *65*, 2697−2702.

(8) Etoumi, A. *J. Pet. Sci. Eng.* **2007**, *55*, 111−121.

(9) Hlatki, M.; Mecs, I.; Puskas, S.; Kalman, M.; Lengyel, G. *U.S. Patent Office*. 2006, Patent No. 0060350.

(10) Zhang, F.; She, Y. H.; Ma, S. S.; Hu, J. M.; Banat, I. M.; Hou, D. J. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 1413−1422.

(11) Zhang, F.; She, Y. H.; Li, H. M.; Zhang, X. T.; Shu, F. C.; Wang, Z. L.; Yu, L. J.; Hou, D. J. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 811−821.

(12) Yi, S. J.; Tan, Y. *J. Oil Gas Technol. (J. JPI)* **2008**, *30*, 144−146.

(13) Chakravarthy, S. S.; Pande, S.; Kapoor, A.; Nerurkar, A. S. *Appl. Biochem. Biotechnol.* **2011**, *165*, 260−269.

(14) Lu, H.; Chandran, K. *Environ. Sci. Technol.* **2010**, *44*, 8943−8949.

(15) Khardenavis, A. A.; Kapley, A.; Purohit, H. J. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 403−9.

(16) Shen, Z.; Zhou, Y.; Hu, J.; Wang, J. *J. Hazard Mater.* **2013**, *15*, 431−438.

(17) Magot, M.; Ollivier, B.; Patel, B. K. C. *Antonie van Leeuwenhoek* **2000**, *77*, 103−116.

(18) Katsivela, E.; Moore, E. R.; Maroukli, D.; Strömpl, C.; Pieper, D.; Kalogerakis, N. *Biodegradation* **2005**, *16*, 169−180.

(19) Toledo, F. L.; Gonzalez-Lopez, J.; Calvo, C. *Bioresour. Technol.* **2008**, *99*, 8470−8475.

(20) Neria-González, I.; Wang, E. T.; Ramírez, F.; Romero, J. M.; Hernández-Rodríguez, C. *Anaerobe* **2006**, *12*, 122−33.

(21) Agrawal, A.; Vanbroekhoven, K.; Lal, B. *Anaerobe* **2010**, *16*, 12−8.

(22) Ren, H. Y.; Zhang, X. J.; Song, Z. Y.; Rupert, W.; Gao, G. J.; Guo, S. X.; Zhao, L. P. *PloS One* **2011**, *6*, e23258.

(23) She, Y. H.; Zhang, F.; Xiang, T. S.; Liu, B. B.; Zhao, L. P. *Acta Ecol. Sin.* **2005**, *25*, 237−242.

(24) Davis, J. P.; Struchtemeyer, C. G.; Elshahed, M. S. *Microb. Ecol.* **2012**, *64*, 942−54.

(25) Banat, I. M. *Bioresour. Technol.* **1995**, *51*, 1−12.

(26) Amani, H.; Müller, M. M.; Syldatk, C.; Hausmann, R. *Appl. Biochem. Biotechnol.* **2013**, *17*, 1080−1093.

(27) Perfumo, A.; Banat, I. M.; Canganella, F.; Marchant, R. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 132−138.

(28) Wang, Q.; Fang, X.; Bai, B.; Liang, X.; Shuler, P. J.; Goddard, W. A.; Tang, Y. *Biotechnol. Bioeng.* **2007**, *98*, 842−853.

(29) Joshi, S.; Bharucha, C.; Desai, A. J. *Bioresour. Technol.* **2008**, *99*, 4603−4608.

(30) Saikia, R. R.; Deka, S.; Deka, M.; Banat, I. M. *Ann. Microbiol. (Heidelberg, Ger.)* **2012**, *62*, 753−763.

(31) Yan, P.; Lu, M.; Yang, Q.; Zhang, H. L.; Zhang, Z. Z.; Chen, R. *Bioresour. Technol.* **2012**, *116*, 24−28.

(32) Sadeghazad, A.; Ghaemi, N. *SPE* **2003**, 80529.

(33) Sahoo, S.; Datta, S.; Biswas, D.; Banik Choudhury, R. *J. Oleo Sci.* **2010**, *59*, 601−605.

(34) Cerqueira, V. S.; Hollenbach, E. B.; Maboni, F.; Vainstein, M. H.; Camargo, F. A.; do Carmo, R.; Peralba, M.; Bento, F. M. *Bioresour. Technol.* **2011**, *102*, 11003−11010.

(35) Thavasi, R.; Jayalakshmi, S.; Banat, I. M. *Bioresour. Technol.* **2011**, *102*, 772−778.

(36) Ohno, K.; Maezumi, S.; Sarma, H. K.; Enomoto, H.; Hong, C.; Zhou, S. C.; Fujiwara, K. *SPE* **1999**, 54328.

(37) Nagase, K.; Zhang, S. T.; Asami, H.; Yazawa, N.; Fujiwara, K.; Enomoto, H.; Hong, C. X.; Laing, C. X. *SPE* **2001**, 68720.

(38) Saadoun, I. *J. Basic Microbiol.* **2002**, *42*, 420−428.

(39) Marchant, R.; Banat, I. M. *Biotechnol. Lett.* **2012**, *34*, 1597−1605.

(40) Marchant, R.; Banat, I. M. *Trends. Biotechnol.* **2012**, *30*, 558−565.

(41) Rabiei, A.; Sharifinik, M.; Niazi, A.; Hashemi, A.; Ayatollahi, S. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 5979−5991.

(42) Sarafzadeh, P.; Hezave, A. Z.; Ravanbakhsh, M.; Niazi, A.; Ayatollahi, S. *Colloids. Surf., B* **2013**, *105*, 223−229.